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Title: High salinity in molasses wastewaters shifts anaerobic digestion to carboxylate production

Running title: Carboxylate production during molasses fermentation

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## Abstract

Biorefinery wastewaters are often treated by means of anaerobic digestion to produce biogas. Alternatively, these wastewaters can be fermented, leading to the formation of carboxylates. Here, we investigated how lab-scale upflow anaerobic sludge blanket reactors could be shifted to fermentation by changing organic loading rate, hydraulic retention time, pH, and salinity. A strong increase in volatile fatty acid concentration up to 40 g COD L<sup>-1</sup> was achieved through increasing salinity above 30 mS cm<sup>-1</sup>, as well as a decrease in methane production by more than 90%, which could not be obtained by adjusting the other parameters, thus, indicating a clear shift from methane to carboxylate production. Microbial community analysis revealed a shift in bacterial community to lower evenness and richness values, following the increased salinity and VFA concentration during the fermentation process. A selective enrichment of the hydrogenotrophic Methanomicrobiales took place upon the shift to fermentation, despite a severe decrease in methane production. Particle size distribution revealed a strong degranulation of the sludge in the reactor, related to the high salinity, which resulted in a wash-out of the biomass. This research shows that salinity is a key parameter enabling a shift from methane to carboxylate production in a stable fermentation process.

**Keywords** carboxylate production; fermentation; methane; microbial community; salinity

## 1. Introduction

A transition to a more sustainable bio-economy moves the production of building block chemicals away from fossil fuel resources towards renewable biological resources at the biorefinery (Choi et al. 2015, McCormick and Kautto 2013, Parajuli et al. 2015). The success of biorefineries strongly depends on their ability to maximize resource and energy recovery from the initial biomass (Moraes et al. 2015). This means considering also the waste streams that are generated during the production process. For example, bio-ethanol production by wet milling generates up to 20 litres of wastewater per litre of bio-ethanol (Ryan et al. 2009, Satyawali and Balakrishnan 2008, Yan et al. 2012). These wastewaters require adequate treatment to ensure the sustainability of the plant in terms of maximizing energy and resource recovery, and preventing environmental pollution of the receiving water bodies (Moraes et al. 2015, Ryan et al. 2009, Satyawali and Balakrishnan 2008).

Anaerobic digestion can be considered a suitable technology to treat these wastewaters, because their high organics content allows efficient biogas and subsequent energy recovery under the form of electricity and heat (Satyawali and Balakrishnan 2008, Syutsubo et al. 2013). The often high salinity and high ammonia concentration in these wastewaters has been shown to cause failure of methanogenesis, requiring dilution and/or co-digestion with other substrates to alleviate inhibition (De Vrieze et al. 2014b, De Vrieze et al. 2015b, Fang et al. 2011). Moreover, biogas requires CO<sub>2</sub> and H<sub>2</sub>S removal before it can be distributed on the natural gas network (Kleerebezem and van Loosdrecht 2007, Poschl et al. 2010). Assuming an electrical efficiency of 35-40% of a combined heat and power system (Deublein and Steinhauser 2008), and taking into account a market price of € 0.10 kWh<sup>-1</sup> for electricity and € 0.05 kWh<sup>-1</sup> for heat (Verstraete and Vlaeminck 2011), 1 kg COD converted to biogas corresponds to only € 0.20-0.25.

Instead of completely degrading the COD in the biorefinery wastewaters to CH<sub>4</sub> and CO<sub>2</sub> for

energy generation, the production of carboxylates by a fermentation process could be a suitable alternative, falling within the carboxylate platform (Agler et al. 2011). Fermenting bacteria have a lower sensitive to high ammonia and high salt concentrations, compared to methanogenic archaea (Chen et al. 2008, De Vrieze et al. 2012), thus, allowing higher loading rates and/or eliminating the need for dilution or co-fermentation. In addition, the resulting carboxylates have an intrinsic higher economic value than biogas. Assuming a market price of € 600-800 per ton for crude acetate, 1 kg of COD in the wastewater converted to acetate corresponds to €0.55-0.75, which is at least two times higher than the value of biogas. Longer chain fatty acids, such as butyrate and caproate, can, generally, reach even higher market prices (Posada et al. 2013). However, to obtain carboxylates with a sufficient level of purity, three major barriers need to be solved: product separation from the fermentation broth, product specificity, and methanogenesis inhibition (Agler et al. 2011). Product separation can take place via several techniques, such as membrane electrolysis, whether or not in combination with phase separation (Andersen et al. 2014, Xu et al. 2015). Product specification requires the adjustment of operational parameters to select for those micro-organisms producing the desired compounds (Agler et al. 2011, Vanwonterghem et al. 2014). However, before product specification can be obtained, the methanogens need to be (selectively) inhibited to prevent carbon losses via methane.

The objective of this research was to evaluate whether adjusting (1) the organic loading rate (OLR), (2) hydraulic retention time (HRT), (3) pH, or (4) salt concentration could shift anaerobic digestion to fermentation by a (selective) inhibition of methanogenesis in an upflow anaerobic sludge blanket (UASB) reactor. These operational parameters were selected because of their substantial influence on the methanogenesis process (De Vrieze et al. 2012). Moreover, this strategy does not require the addition of costly chemicals that could specifically inhibit methanogenesis, which are often also affecting other micro-organisms

95 essential for carboxylate production (Liu et al. 2011). Molasses wastewater was selected as a  
96 *proxy* of biorefinery wastewaters, because of its inherent high salinity, and both biogas and  
97 carboxylate production were monitored to evaluate whether methanogenesis inhibition took  
98 place.  
99

## 2. Materials and Methods

### 2.1. Inoculum and substrate characterization

The granular sludge used as inoculum for experiment 1 was obtained from the UASB digesters of a previous experiment in which molasses wastewaters were used for methane production (Desloover et al. 2015). The reactors in experiment 2 were seeded with granular sludge from a full-scale UASB digester (Van Steenberge, Ertvelde, Belgium) in which brewery wastewater was treated at 34°C. The characteristics of both inocula are presented in Table S1. For each experiment a new batch of molasses was obtained from the company AVEBE (Veendam, The Netherlands), and stored at 4 °C until use. The characteristics of both molasses batches are shown in Table 1.

### 2.2. Experimental design

#### 2.2.1. Experiment 1: effect of pH and organic loading rate

Two glass lab-scale UASB reactors with a liquid volume of 2.3 L were operated independently at  $34 \pm 1^\circ\text{C}$  for a period of 127 days. The reactors were inoculated at an initial sludge concentration of 12 g VS L<sup>-1</sup> (volatile solids), and operated as described in SI (S1). The first reactor (UASB-5.5) was set at low set-point of pH 5.5, while the second reactor (UASB-6.5) was set at pH 6.5. The experiment consisted of three subsequent phases. During Phase 1, which lasted from day 0 to 85, the OLR in both reactors was increased up to a value of 10.0 g COD L<sup>-1</sup> d<sup>-1</sup> by decreasing the HRT (Table 2). In Phase 2 (day 86 to 106) the COD concentration in the wastewater was doubled, resulting in an OLR of 20.0 g COD L<sup>-1</sup> d<sup>-1</sup>. Finally, the concentration was set back to the original value of Phase 1 in Phase 3 (day 106 to 127). No sludge was removed during the entire experimental period, and, therefore, the reactors were operating at an infinite high sludge retention time (SRT). The pH was controlled by adjusting the lower level (minimum pH) with a 1M NaOH solution, and

monitored continuously, while biogas production and composition, conductivity, volatile fatty acid (VFA) concentrations, and total and soluble COD were analysed three times per week. In parallel, influent samples were taken three times per week for pH, VFA, conductivity, and total and soluble COD analysis. Gas production was monitored by means of an in-house constructed gas meter based in oil displacement, and reported at standard temperature (273 K) and pressure (101325 Pa) conditions. Samples for cation analysis were taken every 2-3 weeks from the reactors. Samples for DNA extraction were taken from the inoculum and the reactors on day 76 and 127, and stored at -20°C until analysis.

### *2.2.2. Experiment 2: effect of conductivity and HRT*

The same experimental set-up as in experiment 1 was used, though in this case the reactors had a liquid volume of 3.25 L. Two UASB reactors were operated for a period of 75 days without pH control. Both reactors were seeded with inoculum until a sludge concentration of 10 g VS L<sup>-1</sup> was reached. After an adaptation period of 21 days, during which both reactors were fed with the same influent, in one reactor (UASB-HRT) the OLR was increased by decreasing the HRT, keeping the COD concentration in the feed constant, while in the other reactor (UASB-Cond) the OLR was increased by increasing the COD concentration in the feed, keeping the HRT constant (Table 3). Similar to experiment 1, no sludge was removed during the experimental period. Sampling was performed as in experiment 1. Samples for DNA extraction were taken from the inoculum and the reactors on day 75, and stored at -20°C until analysis. Samples for particle size distribution (PSD) analysis were also taken from the inoculum and the reactors on day 75.

### *2.3. Microbial community analysis*

The extraction of DNA was performed following the protocol of Vilchez-Vargas et al. (2013).



The quality of the DNA extracts was evaluated visually by agarose gel electrophoresis. A StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) was used for the real-time PCR analysis. For each DNA extract a 100-fold dilution was made, and analysed in triplicate. The real-time PCR program was carried out as previously described (Desloover et al. 2015), using the primers for the methanogenic populations described by Yu et al. (2005), and total bacteria described by Ovreas et al. (1997). The results were represented as copies per gram of wet sludge. The quality of the real-time PCR analysis was confirmed by the different parameters that were obtained during processing of the data with the StepOnePlus software V2.3 (Table S2).

The DNA extracts were diluted with nuclease-free water to 1 ng DNA  $\mu\text{L}^{-1}$ , and sent to LGC Genomics GmbH (Berlin, Germany) for sequencing. The preparation of the libraries for the Illumina platform (MiSeq), amplicon sequencing, and final data processing were carried out as described in SI (S4).

#### *2.4. Particle size distribution*

Particle size distribution (PSD) analysis of the sludge samples was carried out using a Mastersizer S (Malvern Instruments, Malvern, UK) with a 1000F lens. Before measurement, the sample was added to the MS-17 wet sample dispersion unit to obtain an obscuration between 10 and 30%. The real and imaginary refractive indices for the particles were fixed at 1.596 and 0.100, respectively, whereas the dispersant refractive index was set at 1.333. The results were calculated using the “polydisperse” analysis model in the Mastersizer software, yielding a PSD between 4 and 3500  $\mu\text{m}$ . Each sample was measured in triplicate. A PSD was determined of the inoculum and final sludge of the reactors in experiment 2.

## 2.5. Analytical techniques

Total and volatile solids (TS, VS), total and volatile suspended solids, total ammonia nitrogen (TAN), total Kjeldahl nitrogen (TKN), and total COD were determined according to Standard Methods (Greenberg et al. 1992). The pH was measured using a C532 pH meter (Consort, Turnhout, Belgium), and conductivity was measured by means of a C833 conductivity meter (Consort, Turnhout, Belgium). The free ammonia concentration ( $\text{NH}_3$ ) was calculated from the TAN concentration, pH and temperature in the reactor (Anthonisen et al. 1976). Sodium, potassium, calcium, and magnesium were measured by means of ion chromatography (IC, Metrohm IC 761, Herisau, Switzerland) with a Metrosep C6 – 250/4 column and Metrosep C4 Guard/4.0 guard column. A solution containing 1.7 mM  $\text{HNO}_3$  and 1.7 mM dipicolinic acid was used as eluent. Samples were filtered over a 0.45  $\mu\text{m}$  filter (type PA-45/25, Macherey-Nagel, Germany) to retain solids, and diluted with milli-Q water, if necessary. Biogas composition was analysed by a compact GC (Global Analyser Solutions, Breda, The Netherlands), as described in SI (S5). The different VFA concentrations were measured by gas chromatography, as described in SI (S5).

## 2.6. Statistical analysis

Statistical analysis of the operational data of the two reactors both in experiment 1 and 2 was implemented using the SPSS Statistics 21 software (IBM Corp., Belgium). The normality and homoscedasticity were evaluated by means of the Shapiro-Wilk and Levene test, respectively. A non-parametric Wilcoxon signed-rank test was used to compare the two reactors in both experiments, as normality and homoscedasticity of the operational data could not be confirmed.

### 3. Results

#### 3.1. Effect of pH and organic loading rate on methane and VFA production

During experiment 1 the OLR was increased at similar rates in both reactors, while pH was set a lower set-point of 6.5 in UASB-6.5 and 5.5 in UASB-5.5. The pH remained higher than 7.0 until day 97 of the experiment, due to the fact that VFA production was insufficient to decrease pH (Figure S2). On day 99, fourteen days after the OLR was increased (Table 2), the pH dropped, and was maintained around 6.5 in UASB-6.5, while it only reached 6.3 in UASB-5.5. During the first 97 days, methane production showed a gradual increase, in relation to the increase in OLR, to a maximum value of  $4.17 \text{ L CH}_4 \text{ L}^{-1} \text{ d}^{-1}$  in UASB-6.5 and  $4.69 \text{ L CH}_4 \text{ L}^{-1} \text{ d}^{-1}$  in UASB-5.5. Methane production strongly decreased after day 97 to average values of only  $0.25 \text{ L CH}_4 \text{ L}^{-1} \text{ d}^{-1}$  in UASB-6.5 and  $0.15 \text{ L CH}_4 \text{ L}^{-1} \text{ d}^{-1}$  in UASB-5.5 at the end of the experiment, due to the sudden increase in feed concentration and OLR (Table 2), and was significantly lower ( $P = 0.046$ ) in UASB-5.5 compared to UASB-6.5, although pH was only 0.2 units lower (Figure 1a).

The total VFA concentration remained below a value of  $7.0 \text{ g COD L}^{-1}$  in both reactors, yet, after the increase in the feed concentration on day 86, VFA concentration strongly increased to maximum values of  $32.3 \text{ g COD L}^{-1}$  in UASB-6.5 and  $38.4 \text{ g COD L}^{-1}$  in UASB-5.5 (Figure 2a and b). Although no significant difference ( $P = 0.06$ ) was observed in total VFA concentration between the two reactors, the composition did significantly differ between the two reactors. The fraction of acetate ( $P = 0.019$ ) and propionate ( $P = 0.023$ ) was higher in UASB-6.5, with values of  $44.3 \pm 7.1 \%$  and  $17.6 \pm 4.6 \%$ , respectively, compared to UASB-5.5, with values of  $35.4 \pm 4.0 \%$  and  $13.2 \pm 1.7 \%$ , while butyrate reached a higher ( $P = 0.003$ ) value of  $46.1 \pm 7.0 \%$  in UASB-5.5 (actual pH of 6.3), compared to only  $33.6 \pm 10.4 \%$  in UASB-6.5.

The conductivity, which can be considered a measurement of the salinity in the reactor,

showed no significant differences ( $P = 0.235$  between day 0 and 97 and  $P = 0.125$  between day 98 and 127) between the two reactors, as feed composition was the same (Figure 3a). Conductivity remained below a value of  $30 \text{ mS cm}^{-1}$  until day 86, when an increase in feed concentration resulted in an increase in conductivity up to  $45 \text{ mS cm}^{-1}$ . The elevated conductivity was mainly due to the high potassium concentrations that accumulated up to  $7.87 \text{ g L}^{-1}$  in UASB-6.5 and  $8.20 \text{ g L}^{-1}$  in UASB-5.5 (Figure 4a), while the sodium concentration remained below  $0.5 \text{ g L}^{-1}$ , TAN concentration below  $1.5 \text{ g N L}^{-1}$ , and free ammonia concentration below  $60 \text{ mg N L}^{-1}$  in both reactors (Figure S3, S4 and S5). The increase in conductivity caused sludge granule disintegration and biomass washout (data not shown), which is why the OLR was halved again at day 107 by decreasing the feed concentration, keeping HRT constant (Table 2). This was carried out without modifying the pH or VFA concentration in the feed, but no recovery could be obtained.

### *3.2. Effect of hydraulic retention time and salinity on methane and VFA production*

The results of experiment 1 indicated that an increase in conductivity could be responsible for the inhibition for methanogenesis and subsequent increase in VFA. Experiment 2 was designed to evaluate this. During the first 21 days of the experiment the HRT and salinity were maintained similar in both reactors, which resulted in similar pH (Figure S2) and methane production (Figure 1b) values, with no significant differences ( $P = 0.257$  &  $0.26$ , respectively). After day 21, an increase in molasses concentration in the feed of UASB-Cond, compared to a decrease in HRT in UASB-HRT, resulted in a significant lower ( $P = 0.001$ ) methane production in UASB-Cond (Figure 1b), indicating that a high salinity leads to the inhibition of methanogenesis.

The total VFA concentration was significantly higher ( $P < 0.001$ ) in UASB-Cond with a maximum value of  $39.7 \text{ g COD L}^{-1}$ , compared to UASB-HRT with a maximum value of  $14.7$

g L<sup>-1</sup>, as was the case for the acetate ( $P < 0.001$ ) and butyrate ( $P < 0.001$ ) concentration, yet, propionate concentration was not significantly different ( $P = 0.236$ ) between the reactors (Figure 2c and d). A maximum acetate and butyrate concentration of 13.8 and 24.2 g COD L<sup>-1</sup>, respectively, was obtained in UASB-Cond, while this was only 6.8 and 5.3 g COD L<sup>-1</sup>, respectively, in UASB-HRT. The average acetate fraction from day 21 on was similar in UASB-Cond ( $40.3 \pm 8.3$  %) and UASB-HRT ( $39.6 \pm 7.2$ ), but the propionate fraction was lower in UASB-Cond ( $12.7 \pm 10.2$  %) compared to UASB-HRT ( $26.2 \pm 5.4$  %), while butyrate was higher in UASB-Cond ( $37.5 \pm 17.3$  %) compared to UASB-HRT ( $25.1 \pm 6.3$  %). The conductivity was not significantly different ( $P = 0.26$ ) between the two reactors in the first 21 days of the experiment, yet, when the feeding protocol was changed on day 21, this led to a significant increase ( $P < 0.001$ ) in conductivity in UASB-Cond compared to UASB-HRT (Figure 3b). The maximum conductivity in UASB-HRT was 30.4 mS cm<sup>-1</sup>, while a maximum concentration of 49.5 mS cm<sup>-1</sup> was measured in UASB-Cond. As in experiment 1, the measured conductivity was caused mainly by the high maximum potassium concentrations of 12.24 and 5.42 g L<sup>-1</sup> in UASB-Cond and UASB-HRT, respectively (Figure 4b). The sodium concentration remained below 0.90 g L<sup>-1</sup> in both reactors, while the TAN concentration remained below 1.50 g L<sup>-1</sup> in UASB-HRT and 2.00 g N L<sup>-1</sup> in UASB-Cond (Figure S3 and S4). The free ammonia concentration remained below 40 mg N L<sup>-1</sup> in both reactors. The effect of conductivity on the sludge granule integrity was also observed in this experiment, and is further developed in the following section.

### 3.3. Macroscopic sludge property dynamics

Particle size distribution analysis was carried out of the inoculum sample and the final reactor sludge samples (day 75) of UASB-HRT and UASB-Cond of experiment 2. The inoculum sludge sample consisted of dense granules with an average mass-weighted diameter of  $1290 \pm$

38  $\mu\text{m}$ , while in the final sludge samples this was only  $210 \pm 25 \mu\text{m}$  in UASB-HRT and  $54 \pm 2 \mu\text{m}$  in UASB-Cond (Figure 5). This indicates a strong level of disintegration or degranulation of the sludge in both reactors during the experimental period, yet, the 4-times lower value in UASB-Cond compared to UASB-HRT indicates that granule structure was more affected in UASB-Cond. This higher level of sludge disintegration was also reflected in a higher concentration of volatile suspended solids in UASB-Cond, compared to UASB-HRT (Figure S6). The effluent volatile suspended solids concentration increased from  $1.64 \pm 1.00 \text{ g L}^{-1}$  on day 14 to a maximum value of  $3.25 \pm 0.54 \text{ g L}^{-1}$  on day 75 in UASB-HRT, while a much stronger increase was observed in UASB-Cond, from  $1.41 \pm 0.35 \text{ g L}^{-1}$  on day 14 to  $10.61 \pm 1.94 \text{ g L}^{-1}$  on day 70, indicating wash-out of the sludge.

### 3.4. Microbial community composition

#### 3.4.1. Real-time PCR analysis

Real-time PCR revealed a similar total Bacteria and total methanogens abundance in the inoculum, compared to the final samples of UASB-5.5 and UASB-6.5 of experiment 1 (Figure S7). Within the methanogens, the Methanosaetaceae and Methanobacteriales also maintained similar levels, yet, the Methanomicrobiales showed a slight decrease in UASB-6.5 and UASB-5.5, compared to the inoculum (Figure 6).

In experiment 2, UASB-Cond and UASB-HRT reactor samples showed a 10-fold increase, both in total bacteria and total methanogens abundance, compared to the inoculum, yet, no difference between the two reactors was observed (Figure S7). However, the methanogenic groups greatly differed between the two reactors in the reactor sludge at the end of the experiment, as Methanomicrobiales abundance increased to value of  $2.7 \times 10^9 \pm 2.5 \times 10^8$  copies  $\text{g}^{-1}$  in UASB-Cond, while this was only  $5.6 \times 10^7 \pm 1.0 \times 10^7$  copies  $\text{g}^{-1}$  in UASB-HRT (Figure 6). In contrast, both Methanosaetaceae and Methanobacteriales reached higher values

in UASB-HRT, compared to UASB-Cond.

The methanogenic community in UASB-HRT was similar in the reactor sludge, compared to the sludge present in the effluent. In contrast, UASB-Cond had a much higher Methanomicrobiales abundance of  $2.7 \times 10^9 \pm 2.5 \times 10^8$  copies g<sup>-1</sup> in the reactor sludge, while only  $1.0 \times 10^8 \pm 1.0 \times 10^7$  copies g<sup>-1</sup> were observed in the effluent, indicating a clear difference in methanogenic community composition between reactor sludge and effluent in UASB-Cond, which was also confirmed by principal coordinate analysis and hierarchal clustering of the Bray-Curtis dissimilarity indices of the methanogenic community (Figure S8 and S9). No Methanosarcinaceae were detected in any of the samples.

#### 3.4.2. Bacterial community analysis based on 16S rRNA gene sequences

Bacterial community of the inoculum and final reactor sludge samples of UASB-Cond and UASB-HRT resulted in a total of 12,307 reads and 290 different operational taxonomic units (OTUs). Rarefaction curves were generated to evaluate coverage of the bacterial community (Figure S10). Bacterial richness was clearly lower in UASB-Cond (82 OTUs), compared to UASB-HRT (120 OTUs) and the inoculum (142 OTUs). In addition, bacterial diversity was similar in UASB-HRT and the inoculum, but lower in UASB-Cond, based on both the Shannon (3.84 for the Inoculum, 2.57 for UASB-Cond and 3.46 for UASB-HRT) and Simpson (0.94 for the Inoculum, 0.83 for UASB-Cond and 0.93 for UASB-HRT) indices of diversity. The bacterial community in each of the three samples completely differed from each other, based on the Bray-Curtis dissimilarity indices of the relative OTU abundances. A similarity of only 1.9 and 3.8 % was observed between the inoculum and the final samples of UASB-Cond and UASB-HRT, respectively, but also only 12.8 % similarity was observed between UASB-Cond and UASB-HRT, indicating a complete change in bacterial community composition in the two reactors, in relation to each other and the inoculum. The dominant

323 bacterial populations belonged to the Actinobacteria, Bacteroidetes, Firmicutes and  
324 Proteobacteria phyla, yet, clear difference were observed between the samples (Figure 7). In  
325 the inoculum samples, there was a main dominance of Proteobacteria, represented by OTU2  
326 (*Sulfurovum*, 20.7 %) and OTU16 (*Methylobacter*, 5.3 %). In contrast, both UASB-Cond and  
327 UASB-HRT were dominated by Bacteroidetes and Firmicutes. The main OTUs in UASB-  
328 Cond were OTU1 (unclassified Enterococcaceae, 36.8 %), OTU5 (unclassified  
329 Porphyromonadaceae, 12.2 %), OTU6 (unclassified Bacteroidales, 9.5 %), and OTU7  
330 (*Clostridium*, 9.3 %), while the main OTUs in UASB-HRT were OTU3 (unclassified  
331 Bacteroidetes, 19.6 %), OTU4 (*Desulfovibrio*, 9.1 %) and OTU8 (*Olsenella*, 8.7 %).  
332



## 4. Discussion

An increase in OLR did not strongly affect methanogenesis, yet, an increase in overall salinity to a value higher than 30 mS cm<sup>-1</sup> resulted in a complete inhibition of methanogenesis. This also affected granular sludge stability, which resulted in a wash-out of the sludge and a clear shift in microbial community composition. A similar methanogenic community was observed in the effluent and reactor sludge of UASB-HRT, while there was a clear enrichment of Methanomicrobiales in the reactor sludge of UASB-Cond, compared to the effluent.

### *4.1. Salinity is the key parameter for selective inhibition of methanogenesis*

A strong increase in OLR usually results in an uncoupling of the acidogenesis/acetogenesis and methanogenesis process, related to the low growth rate of the methanogens, which results in the accumulation of VFA and a subsequent decrease in pH (De Vrieze et al. 2012, Gujer and Zehnder 1983). A mere increase in OLR by decreasing the HRT, keeping all other parameters constant, did not result in a complete inhibition of methanogenesis. In UASB-5.5, UASB-6.5 and UASB-Cond inhibition of methanogenesis was achieved by increasing the OLR, but only by increasing the molasses feed concentration. The main issue related to many biorefinery wastewaters, which contain high salt concentrations and high buffering capacity, is that VFA production does not cause a decrease in pH to suboptimal values for methane production. In these experiments, the pH did not decrease below a value of 6.3 in any of the reactors, as observed earlier for anaerobic digesters treating molasses wastewaters that experienced severe VFA accumulation (De Vrieze et al. 2014a, De Vrieze et al. 2014b). Although a pH value of 6.3 is outside of the optimal range for methanogens (Appels et al. 2008, Gujer and Zehnder 1983), it was insufficient to permanently and/or completely inhibit them (De Vrieze et al. 2015a, Goux et al. 2015, Regueiro et al. 2015, Staley et al. 2011). An increase in feed concentration, which caused an increase in salinity in the reactor, did inhibit

methanogenesis, as observed in UASB-5.5, UASB-6.5 and UASB-Cond. The shift from methanogenesis to carboxylate production in each of these reactors coincided with the increase of conductivity above the apparent threshold level of 30 mS cm<sup>-1</sup> (De Vrieze et al. 2012). The elevated potassium concentration in the molasses wastewaters might have resulted in an osmotic stress to the methanogens, which caused the inhibition of methane production (Feijoo et al. 1995, Oh et al. 2008). In addition, a decrease in conductivity below this threshold level, as carried out in UASB-5.5 and UASB-6.5 did not result in a revival of methanogenesis. Hence, long-term inhibition of methanogenesis, resulting in stable carboxylate production could be obtained by increasing overall conductivity above a value of 30 mS cm<sup>-1</sup>.

#### *4.2. From methanogenesis to carboxylate production: a shift in bacterial community and selective retention of Methanomicrobiales*

A strong difference in bacterial community composition was observed in UASB-Cond, compared to UASB-HRT and the inoculum sample. This is in spite of the fact that anaerobic digestion, with the exception of methanogenesis, contains the same process steps as fermentation. Thus, the operational conditions related to fermentation, increased salinity and VFA concentrations, determined the outcome of the bacterial community composition (De Vrieze et al. 2015c, Krakat et al. 2011, Town et al. 2014). The shift from methane to carboxylate production was also associated with a decrease in bacterial evenness and richness, which can be considered a reflection of more selective conditions for the microbial community involved in the process, and potentially lower functional resilience (Carballa et al. 2015, Goux et al. 2015, Werner et al. 2011, Wittebolle et al. 2009). The difference in bacterial community between UASB-Cond and UASB-HRT was also reflected in the methanogenic community. The UASB-HRT was able to maintain methane

production, with the exception of the last 15 days of the experiment when an OLR of  $24.0 \pm 2.1 \text{ g COD L}^{-1} \text{ d}^{-1}$  was applied. This relates to the increased abundance of Methanosaetaceae and Methanobacteriales of which the former are regarded as crucial for stable methanogenesis in UASB systems (Hulshoff Pol et al. 2004, Kim et al. 2015), and the latter of high importance in high-rate systems (Bialek et al. 2011, De Vrieze et al. 2015c). In contrast, UASB-Cond showed a selective enrichment of Methanomicrobiales in the reactor itself, demonstrated by the fact that effluent sludge had a much lower relative and absolute abundance in Methanomicrobiales. This differed from UASB-HRT in which methanogenic community abundance was similar in the reactor and effluent samples. Methanomicrobiales are, similar to Methanobacteriales, often observed at deteriorating conditions, *i.e.* the accumulation of VFA, in AD (Hao et al. 2013, Leite et al. 2015, Munk et al. 2010). However, while Methanobacteriales could be correlated to biogas production in AD, this was not the case for Methanomicrobiales (De Vrieze et al. 2015c). The selective retention of Methanomicrobiales during high VFA and methane production inhibitory conditions points to an alternative role of Methanomicrobiales, in addition to methane production, and warrants further investigation.

#### *4.3. Towards practical applications to achieve high-rate carboxylate production*

Salinity was demonstrated as a key parameter to shift anaerobic digestion from methanogenesis to fermentation. Considering that waste streams, such as molasses wastewaters have a high salinity to begin with, this may be advantageous towards their conversion to carboxylates. A major problem encountered during the fermentation process was, however, the degranulation of the sludge, which led to a subsequent wash-out of biomass. This could be attributed to the high concentration of monovalent cations, in this case potassium, that could affect the structure of the sludge, as also observed in activated sludge

systems (Higgins and Novak 1997, Kara et al. 2008). This is in contrast with divalent cations that assist in granule formation (Liu et al. 2002, Tiwari et al. 2006). To maintain a sufficient amount of active biomass, and to produce a clean effluent, efficient biomass retention is required. However, the application of liquid waste streams, such as biorefinery wastewaters, require an uncoupling of the sludge retention and hydraulic retention time, as is the case in UASB systems, to maintain high carboxylate production rates. An anaerobic membrane bioreactor system could be a suitable alternative, as both technologies are applied for liquid waste streams, such as biorefinery wastewaters. An other approach could be the application of a pulse in salinity prior to the start-up of the UASB reactor or the addition of divalent cations to inhibit methanogenesis, but to prevent biomass from washing out.

## **5. Conclusions**

We have demonstrated that carboxylate production can be accomplished through the fermentation of biorefinery wastewaters. Salinity served as a key operational parameter to permanently shift anaerobic digestion from methane to carboxylate production. This was reflected in a strong shift in bacterial community and a selective enrichment of Methanomicrobiales. The simplicity of this approach will allow fast application in full-scale systems, yet, it will first require the biomass wash-out barrier to be overcome.

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Data deposition: the sequences reported in this paper have been deposited in the European Nucleotide Archive (ENA) database (Accession numbers LT545685- LT545974).

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657 **Tables:**

658 **Table 1** Characteristics of the two batches of undiluted molasses that were used during  
 659 experiment 1 and experiment 2. Each analysis was carried out in triplicate. BDL = below the  
 660 limit of detection. FW = fresh weight.

Parameter	Unit	Experiment 1	Experiment 2
pH	-	5.68 ± 0.01	5.71 ± 0.05
TS	g TS kg <sup>-1</sup> FW	504.6 ± 0.0	565.8 ± 4.3
VS	g VS kg <sup>-1</sup> FW	342.1 ± 2.5	379.6 ± 3.1
Total COD	g COD kg <sup>-1</sup> FW	445.1 ± 6.8	401.9 ± 7.5
Conductivity	mS cm <sup>-1</sup>	25.7 ± 0.1	26.1 ± 0.4
Total VFA	mg COD kg <sup>-1</sup> FW	5808 ± 256	13960 ± 1042
Acetic acid	mg COD kg <sup>-1</sup> FW	5670 ± 250	3873 ± 243
Propionic acid	mg COD kg <sup>-1</sup> FW	139 ± 6	10048 ± 1013
TAN	g N kg <sup>-1</sup> FW	1.52 ± 0.08	1.51 ± 0.05
TKN	g N kg <sup>-1</sup> FW	20.78 ± 5.20	26.08 ± 0.76
Na <sup>+</sup>	g kg <sup>-1</sup> FW	2.02 ± 0.19	2.66 ± 0.05
K <sup>+</sup>	g kg <sup>-1</sup> FW	72.44 ± 2.08	85.91 ± 2.88
Ca <sup>2+</sup>	g kg <sup>-1</sup> FW	BDL	BDL
Mg <sup>2+</sup>	g kg <sup>-1</sup> FW	3.97 ± 0.34	4.28 ± 0.16
COD: N ratio	-	21.42 ± 5.37	15.41 ± 0.53
TS:VS ratio	-	1.48 ± 0.01	1.49 ± 0.02
COD:VS ratio	-	1.30 ± 0.02	1.06 ± 0.02

661

662 **Table 2** Operational parameters of UASB-5.5, set at low pH 5.5, and UASB-6.5, set at low  
663 pH 6.5. OLR = organic loading rate.

Time (days)	UASB-5.5		UASB-6.5	
	HRT (days)	OLR (g COD L <sup>-1</sup> d <sup>-1</sup> )	HRT (days)	OLR (g COD L <sup>-1</sup> d <sup>-1</sup> )
1-14	5.8 ± 0.7	4.6 ± 0.6	7.4 ± 2.0	3.6 ± 1.0
15-29	4.5 ± 0.3	5.9 ± 0.5	4.5 ± 0.2	5.9 ± 0.5
30-42	3.4 ± 0.3	7.9 ± 0.8	3.2 ± 0.1	8.4 ± 0.6
43-85	2.6 ± 0.2	10.1 ± 1.0	2.7 ± 1.5	9.9 ± 0.8
86-106	2.7 ± 0.2	19.6 ± 2.6	2.8 ± 0.2	19.0 ± 2.5
107-127	3.0 ± 1.0	10.0 ± 1.1	3.0 ± 0.7	9.7 ± 1.1

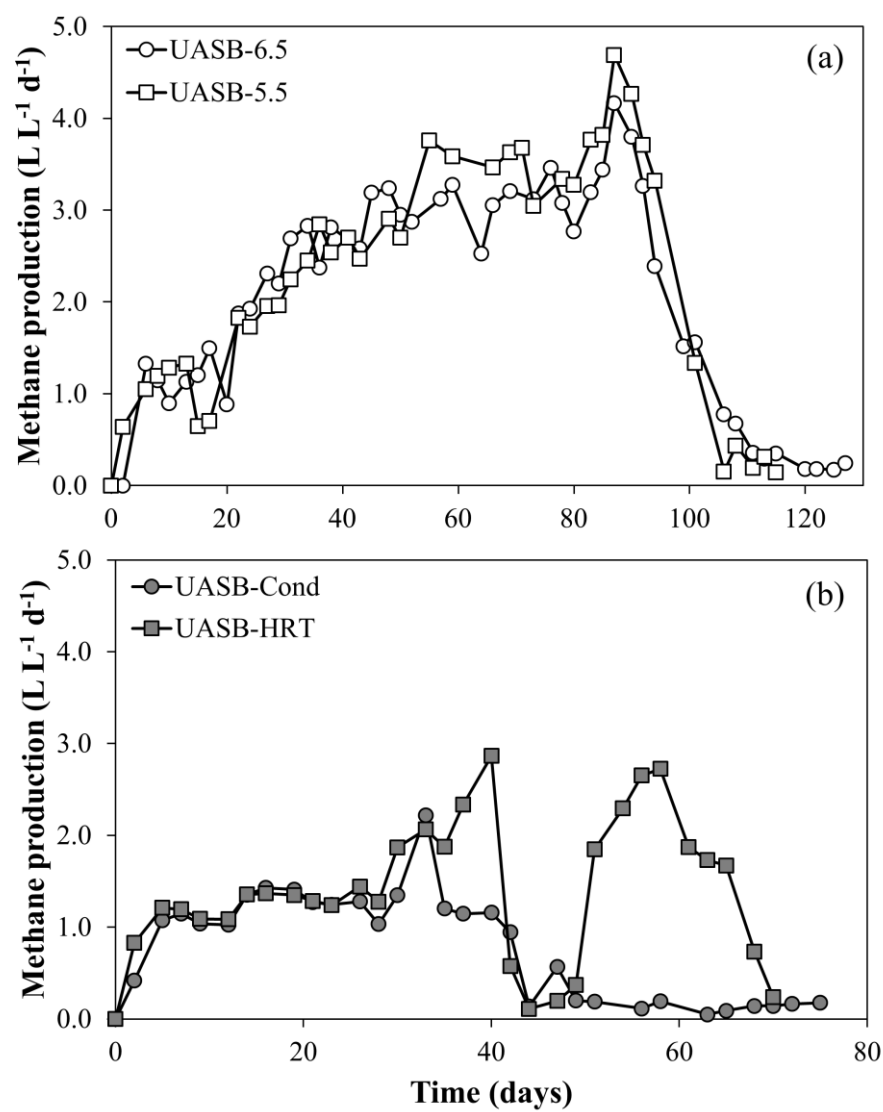
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665 **Table 3** Operational parameters of UASB-HRT, with constant molasses feed concentration,  
666 and UASB-Conductivity, with constant HRT. OLR = organic loading rate.

Time (days)	UASB-HRT		UASB-Conductivity	
	HRT (days)	OLR (g COD L <sup>-1</sup> d <sup>-1</sup> )	HRT (days)	OLR (g COD L <sup>-1</sup> d <sup>-1</sup> )
1-12	6.7 ± 0.1	4.2 ± 0.2	7.2 ± 0.1	4.0 ± 0.2
13-21	4.3 ± 0.1	6.6 ± 0.3	4.2 ± 0.0	6.8 ± 0.4
22-36	2.1 ± 0.2	10.5 ± 1.4	3.0 ± 0.1	9.5 ± 0.6
37-54	1.5 ± 0.1	14.3 ± 1.5	3.3 ± 0.4	12.4 ± 1.7
55-75	1.0 ± 0.0	24.0 ± 2.1	3.6 ± 0.5	15.8 ± 2.8

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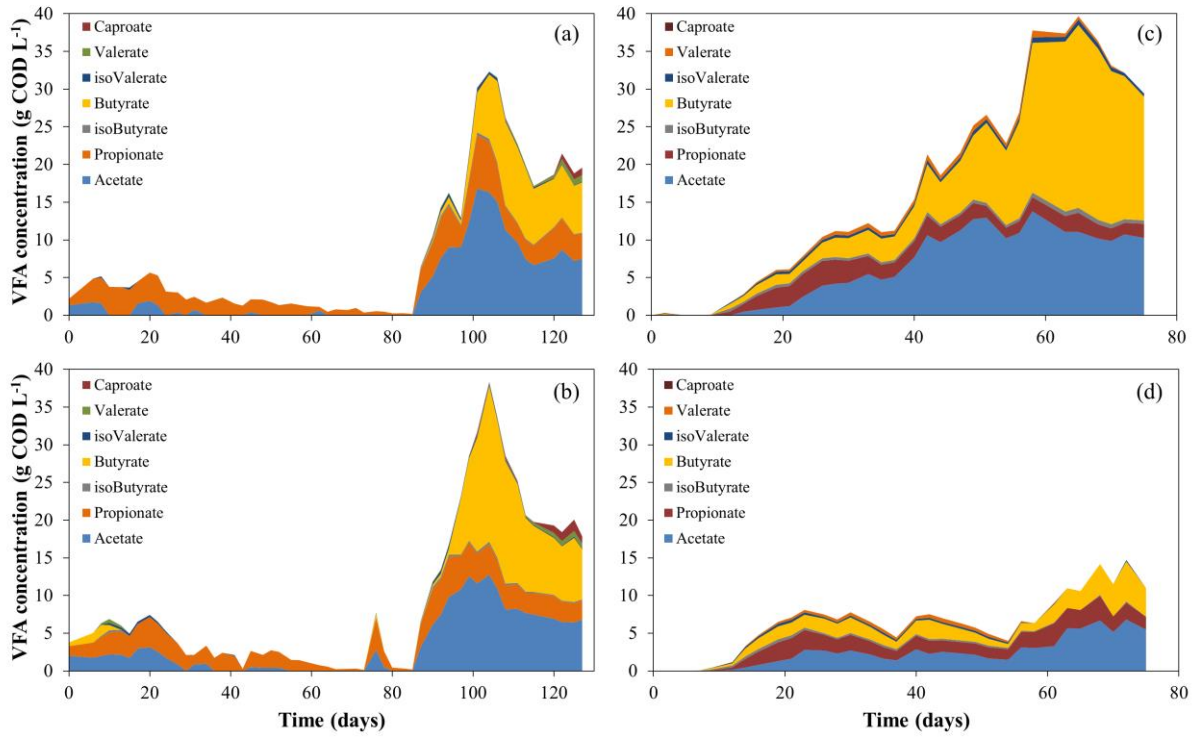
668     Figures:



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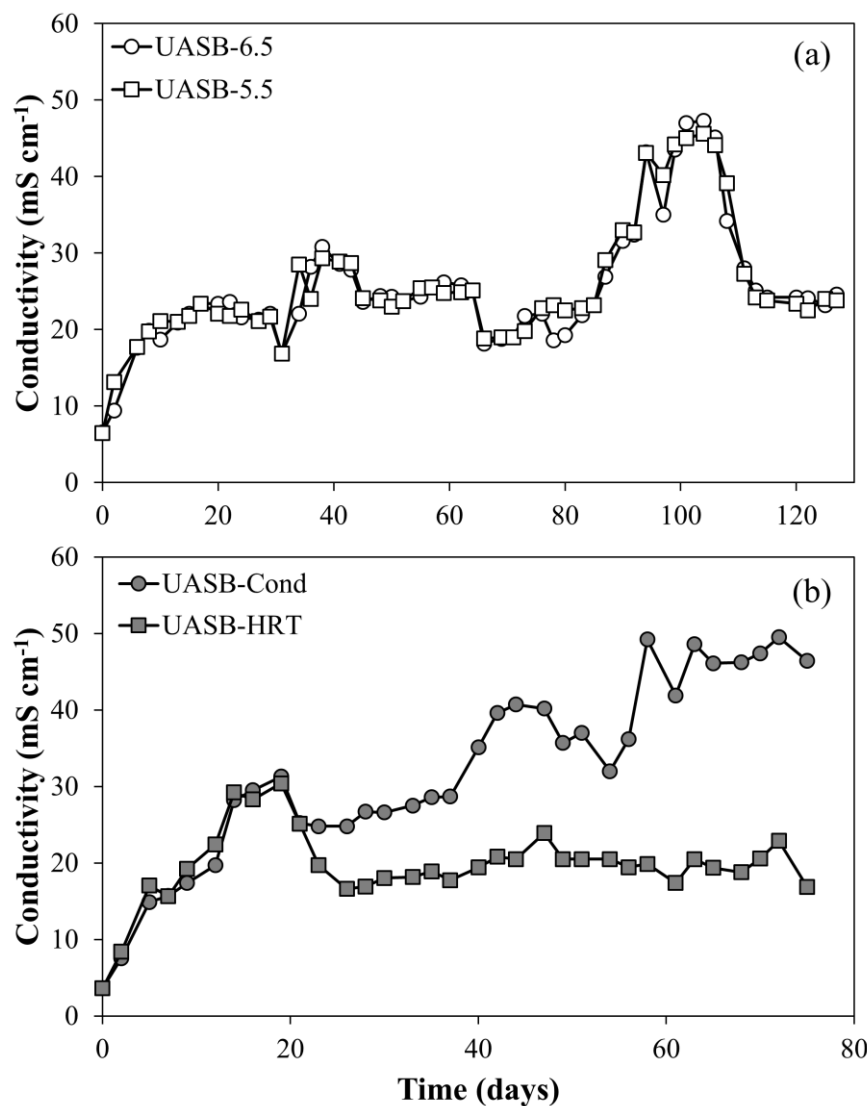
670     **Figure 1** Methane production rate in (a) experiment 1, and (b) experiment 2.

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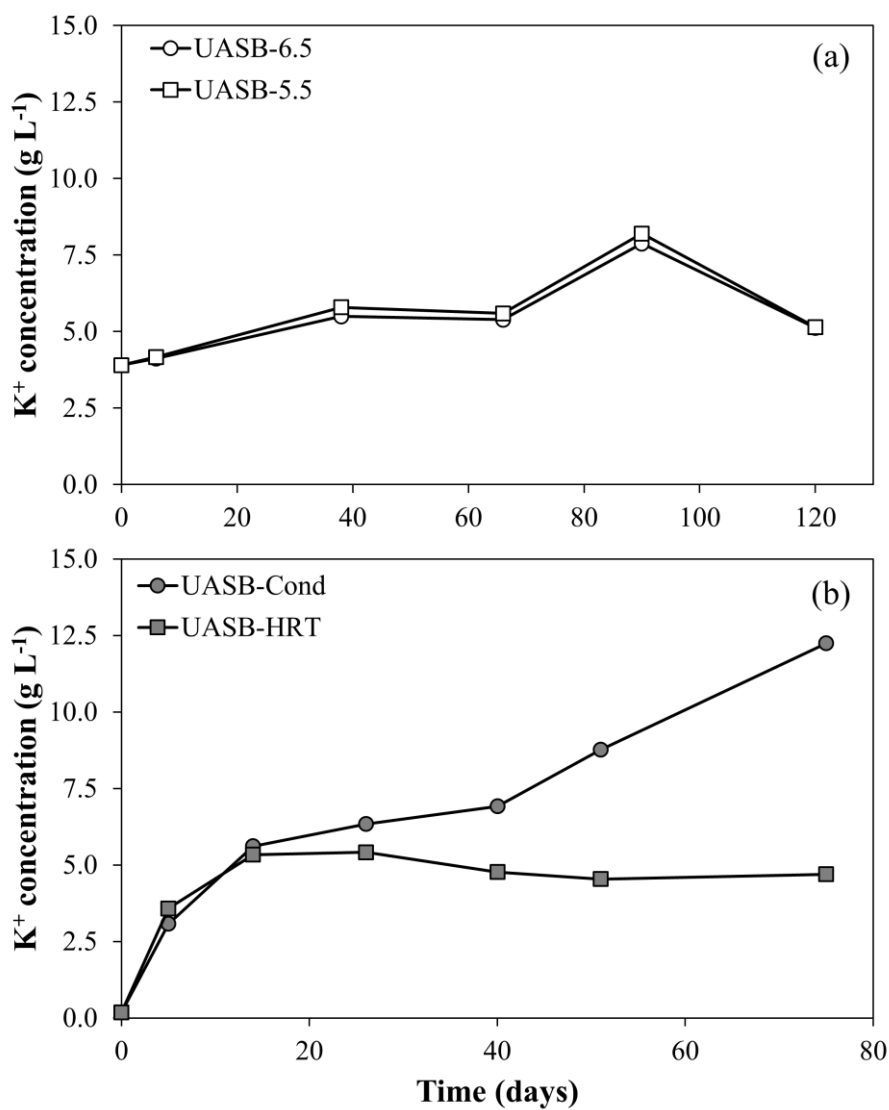


**Figure 2** Volatile fatty acid concentration and composition profiles in (a) UASB-6.5 and (b) UASB-5.5 in experiment 1, and (c) UASB-Cond and (d) UASB-HRT in experiment 2.

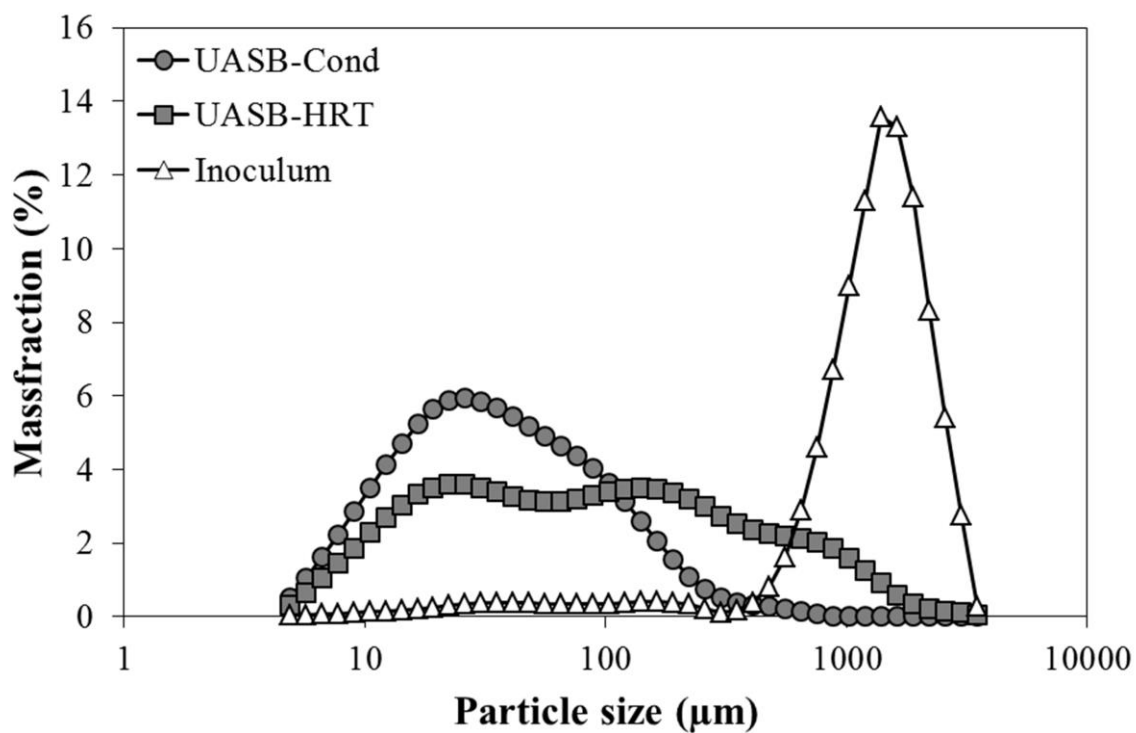




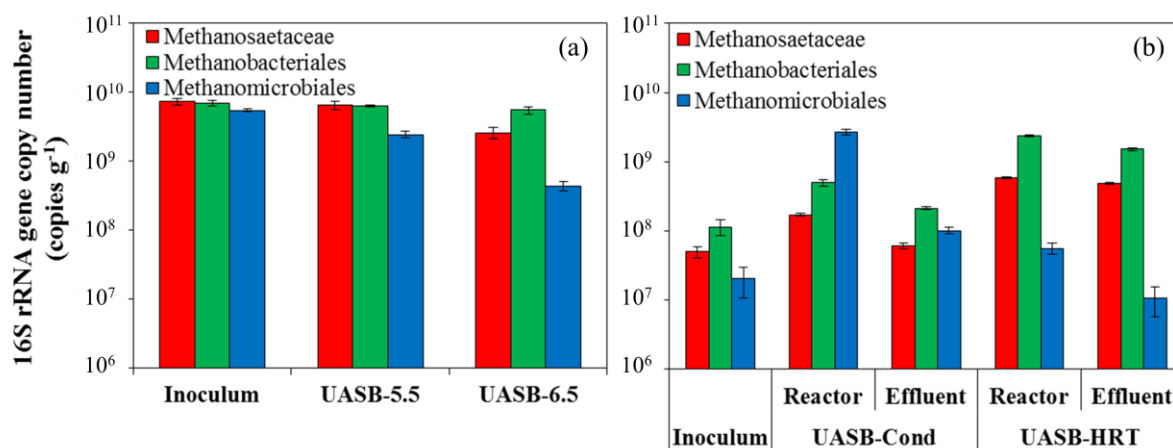
**Figure 3** Conductivity in (a) experiment 1, and (b) experiment 2.



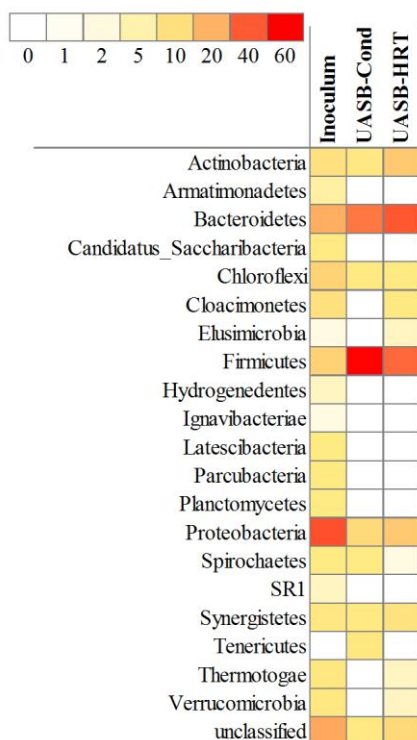
**Figure 4** Potassium concentration in (a) experiment 1, and (b) experiment 2.



**Figure 5** Particle size distribution of the inoculum sludge sample, and the final samples after 75 days of operation in UASB-Cond and UASB-HRT.



**Figure 6** Real-time PCR results of the Methanosaetaceae, Methanobacteriales and Methanomicrobiales in (a) the Inoculum and final reactor samples of UASB-6.5 and UASB-5.5 in experiment 1, and (b) the Inoculum and final reactor and effluent samples of UASB-Cond and UASB-HRT in experiment 2.



**Figure 7** Heatmap representing the bacterial phyla present in at least one of the three samples.

The colour scale ranges from 0 to 60% relative abundance.